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GTP Depletion Induced by IMP Dehydrogenase Inhibitors Blocks RNA-Primed DNA Synthesis

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SUMMARY

Inhibitors of IMP dehydrogenase (EC 1.2.1.14), including mizoribine (Bredinin) and mycophenolic acid, have significant antitumor and immunosuppressive activities. Studies were aimed at determining the mechanism by which intracellular GTP depletion induced by these agents results in inhibition of DNA synthesis. Incubation of human CEM leukemia cells for 2 hr with IC_{50} concentrations of either mizoribine (4 μM) or mycophenolic acid (0.5 μM) reduced cellular GTP levels an average of 68% or 58%, respectively, compared with the levels in control cells. Under similar conditions, mizoribine and mycophenolic acid decreased the amount of [^{3}H]adenosine incorporated into primer RNA by 75% and 70%, respectively, relative to the untreated controls, but had no significant effect on total RNA synthesis. Repletion of the guanine nucleotide pools by coincubation of CEM cells with guanosine plus 8-aminoguanosine prevented both the inhibition of primer RNA synthesis and the inhibition of tumor cell growth induced by these agents. Additional studies demonstrated that GTP depletion alone was capable of directly inducing inhibition of primer RNA synthesis. Primer RNA synthesis was inhibited an average of 84% in whole-cell lysates that lacked GTP but contained all remaining ribo- and deoxyribonucleoside triphosphates. On an M13 DNA template, RNA-primed DNA synthesis catalyzed by the purified complex of DNA primase (EC 2.7.7.6) and DNA polymerase α (EC 2.7.7.7) was decreased an average of 70% in the absence of GTP, compared with synthesis in the presence of 0.5 mM GTP. These results provide evidence that mizoribine and mycophenolic acid inhibit DNA replication by inducing GTP depletion, which suppresses the synthesis of RNA-primed DNA intermediates.

IMP dehydrogenase catalyzes the first step in the NAD-dependent conversion of IMP to GMP in the *de novo* purine biosynthetic pathway. The overall level of IMP dehydrogenase activity is positively correlated with the growth rates of a variety of normal and neoplastic tissues (1). Inhibitors of IMP dehydrogenase activity impede the growth of cultured cell lines (2, 3) and induce differentiation of HL-60 cells (4–6) and blasts in patients with chronic myelogenous leukemia in blast crisis (7). It is of interest that inhibition of IMP dehydrogenase by a variety of agents results in inhibition of DNA synthesis that is reversible with the specific repletion of guanine nucleotide pools (3). Although both GTP and dGTP pools are reduced after incubation of cells with IMP dehydrogenase inhibitors, several lines of evidence support a primary role for GTP depletion in inducing the inhibition of DNA synthesis (8–10).

In addition to their potential activities as anticancer agents, two IMP dehydrogenase inhibitors have significant immunosuppressive activity in clinical studies. Mizoribine (Bredinin; 4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) and mycophenolate mofetil, the morpholinoethyl ester of mycophenolic acid, are effective as single agents in prolonging the survival of transplanted organs and in inhibiting lymphocyte activation (11–14). Studies on the effects of mizoribine on isolated, activated, human peripheral blood T cells indicate that guanine nucleotide depletion induced by mizoribine treatment results in inhibition of the entry of T cells into S phase of the cell cycle without inhibiting a number of late G_1 events in T cell activation, including the expression of c-Myb, c-Myc, IL-2, and cdc2 kinase (13).

Although it is clear from previous studies that both mizoribine and mycophenolic acid induce intracellular GTP depletion, the mechanism by which this effect leads to inhibition of DNA synthesis is less certain. One possible direct mechanism involves the inhibition of RNA-primed DNA synthesis that results from drug-induced GTP depletion. Cohen *et al.* (8) previously speculated that mycophenolic acid exerts its cytotoxic effect via this mechanism, but the effects of this agent on RNA-primed DNA synthesis were not investigated. The

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ABBREVIATIONS: rNTP, ribonucleoside triphosphate having an unspecified base; dNTP, deoxyribonucleoside triphosphate having an unspecified base; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

DNA polymerase α-primase complex is responsible for the initiation of DNA synthesis at the origins of replication and for the discontinuous DNA synthesis of the lagging strand of the replication fork (15). The primase activity of the complex catalyzes the synthesis of short RNAs of 8–10 nucleotides (primer RNAs), which are then elongated by the replicative DNA polymerase to form RNA-primed DNA (Okazaki) fragments (16). DNA primase, an RNA polymerase, preferentially utilizes GTP and ATP to initiate primer RNA synthesis on natural DNA templates both *in vitro* (17–21) and *in vivo* (22–24). This enzyme is a target for the antileukemia drug fludarabine phosphate but is not inhibited by other antimetabolites that interfere with DNA synthesis (25, 26).

In an effort to determine the mechanism underlying the block of DNA synthesis induced by mizoribine and mycophenolic acid, we asked whether these IMP dehydrogenase inhibitors could interfere with the DNA-priming activity of the DNA polymerase α-primase complex by depleting the cells of GTP. We examined the consequences of guanine nucleotide depletion on primer RNA formation in CCRF-CEM T lymphocytic leukemia cells and on DNA primase activity purified from these cells.

Experimental Procedures

Materials. Human leukemia cells (CCRF-CEM) were grown at 37° under 95% air/5% CO_2, in Fischer's medium supplemented with 10% heat-inactivated horse serum, 20,000 units/liter penicillin, and 20 mg/liter streptomycin. Tissue culture medium, serum, antibiotics, and the pBR322 plasmid were obtained from GIBCO-BRL (Grand Island, NY). The cells were periodically checked for *Mycoplasma* contamination with a Gen-probe *Mycoplasma* rRNA hybridization kit obtained from Fischer Scientific Co. (Raleigh, NC). Mizoribine was obtained from Asahi Chemical Industry Co. (Tokyo, Japan). Mycophenolic acid, guanosine, 8-aminoguanosine, M13mp8(+) single-stranded DNA, proteinase K, and all of the rNTPs and dNTPs were purchased from Sigma Chemical Co. (St. Louis, MO). RNase-free pancreatic DNase I and RNase A were obtained from Worthington Biochemical Co. (Freehold, NJ). T4 polynucleotide kinase and restriction enzymes were purchased from Promega (Madison, WI). Oligonucleotide size markers were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ) and were labeled at their 5′ ends with [γ-^{32}P]ATP, as described previously (27). [α-^{32}P]dATP and [γ-^{32}P]ATP (specific radioactivities, 3000 Ci/mmol) were purchased from DuPont-NEN Radiochemicals (Boston, MA). [2-^{14}C]Thymidine, [*methyl*-^{3}H]thymidine, and [2,8-^{3}H]adenosine (specific radioactivities, 50, 65, and 36 Ci/mmol, respectively) were purchased from Moravek Biochemicals (Brea, CA).

Cell growth inhibition assay. Exponentially growing CEM cells were incubated for 72 hr in either the absence or the presence of varying concentrations of either mizoribine or mycophenolic acid. Some cell cultures were supplemented with 50 μM guanosine and 100 μM 8-aminoguanosine to replenish the intracellular pool of GTP (13). The numbers of viable cells were then determined either by trypan blue exclusion or with the MTT assay.

rNTP pool measurements. Exponentially growing CCRF-CEM cells (2×10^7) were incubated in the absence or presence of various concentrations of either mizoribine or mycophenolic acid, with or without 50 μM guanosine and 100 μM 8-aminoguanosine. After 2 hr the cells were extracted with perchloric acid, and the extract was assayed for GTP and ATP by high performance liquid chromatography, as described by Turka *et al.* (13).

Uptake of [^{3}H]adenosine. Exponentially growing cells were incubated for 2 hr with either no drug, 2 μM mizoribine, or 0.5 μM mycophenolic acid. The cells were then resuspended in fresh medium (minus serum) at 37° and incubated with 10 μM [^{3}H]adenosine for either 0, 2.5, 5, 10, 20, or 30 min. Cellular uptake of [^{3}H]adenosine into the trichloroacetic acid-soluble pool was then measured as reported previously (28).

Analysis of internucleosomal DNA cleavage. Exponentially growing CEM cells (1×10^6) were incubated for various times in the absence or presence of IC_{50} concentrations of either mizoribine (4 μM) or mycophenolic acid (1 μM), with or without 50 μM guanosine and 100 μM 8-aminoguanosine. Low molecular weight DNA was isolated after lysis of cells with sodium dodecyl sulfate and precipitation of high molecular weight DNA as described by Cohen and Duke (29). The low molecular weight DNA in the supernatant was treated with RNase, extracted with phenol/chloroform, and precipitated. DNA fragments were separated in a 1.5% agarose gel and stained with ethidium bromide. High molecular weight DNA fragments were separated and analyzed by agarose gel electrophoresis as described by Barry and Eastman (30).

Measurement of RNA and DNA synthesis. Exponentially growing CEM cells (three flasks/group) were incubated for 2 hr at 37° in either the absence or presence of varying concentrations of either mizoribine or mycophenolic acid. The incorporation of [^{3}H]adenosine and [^{3}H]thymidine into RNA and DNA, respectively, was determined as described previously (31). Results were expressed as the percentage of the control ^{3}H radioactivity incorporated into either RNA or DNA/1×10^6 cells.

Isolation of RNA-primed DNA from whole cells. CEM cells (4×10^7 cells/group) were preincubated for 72 hr with 0.02 μCi/ml [^{14}C]thymidine and then grown for 24 hr in fresh medium, at a density of 2×10^5 cells/ml. Mycophenolic acid or mizoribine, either alone or in combination with guanosine plus 8-aminoguanosine, was then added and the cells were incubated for an additional 2 hr. At the end of the 2-hr incubation, the cells were centrifuged at 4°, resuspended in 8 ml of fresh prewarmed medium, and labeled with 6.25 μCi/ml [^{3}H]adenosine for 30 min. Nuclei were isolated from the control and drug-treated cells and the nucleic acids were quantitatively extracted from the nuclear matrices as described previously (32). This and all subsequent procedures were carried out under RNase-free conditions. The purified nucleic acids were then centrifuged to equilibrium at $101{,}000 \times g$, in a cesium chloride gradient. Because RNA-primed DNA consists of an oligoribonucleotide covalently attached to nascent DNA of about 100–200 nucleotides, it bands in a cesium chloride gradient at a density similar to that of DNA (1.72–1.75 g/ml), which is lower than that of the bulk [^{3}H]RNA (31). Gradient fractions of 0.45 ml were collected from the bottoms of the tubes, and the amount of radioactivity in a 50-μl aliquot of each fraction was determined by liquid scintillation counting. To further purify the RNA-primed DNA, the fractions collected from the DNA density region of the gradient were pooled and centrifuged in a second cesium chloride gradient. The relative amount of primer RNA synthesized in each sample was then calculated from the area under the curve of ^{3}H radioactivity in the DNA density region of the gradient.

Measurement of primer RNA synthesis in cell lysates. Groups of 2×10^7 exponentially growing CEM cells were prelabeled for 72 hr with 0.05 μCi/ml [^{3}H]thymidine, and whole-cell lysates were prepared as described previously (31). One-milliliter aliquots of the cell lysates were mixed with 0.25 ml of incorporation buffer. Final concentrations in the reaction mixtures were 40 mM NaCl, 5 mM $MgCl_2$, 50 mM sucrose, 30 mM HEPES, 0.4 mM $CaCl_2$, 5 mM phosphoenolpyruvate, 0.8 mM dithiothreitol, 100 μM concentrations of the four dNTPs, 1 μM [α-^{32}P]ATP, and 1 mM UTP and CTP, at a final pH of 7.8. To one group of samples GTP was added to yield a final concentration of 1 mM. After a 10-min incubation at 37°, the nuclei were prepared and the RNA-primed DNA was isolated from the nuclear matrices as described above. The RNA-primed DNA was then digested with 12 units/μl RNase-free DNase I for 22 hr at 37°, heated at 90° in 7 M urea for 5 min, and then cooled on ice for 5 min before loading of the sample on a 20% polyacrylamide gel. Electrophoresis and autoradiography were carried out as described (31).

Oligo- and polynucleotide size markers consisted of [5′-^{32}P](A)$_3$ and a ladder of repeating [5′-^{32}P]d(GACT)$_{8-32}$. The relative amounts of primer RNA synthesized in the presence or absence of GTP were determined by scanning the autoradiographic film with a laser densitometer (LKB, Bromma, Sweden).

Measurement of primase-dependent DNA polymerase α activity. The DNA polymerase α-primase complex was purified from exponentially growing CCRF-CEM cells by immunoaffinity chromatography using the SJK 132–20 monoclonal antibody to DNA polymerase α (26). The enzyme preparation was devoid of RNase activity. One unit of DNA primase or DNA polymerase α activity is defined as the quantity of enzyme required to catalyze the formation of 1 pmol of acid-insoluble product/min on a poly(dT) or activated calf thymus DNA template, respectively. Primase-dependent polymerase α activity was measured as a function of GTP concentration. The reaction mixtures contained 50 mM Tris·HCl, pH 8.0, 8 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml heat-inactivated bovine serum albumin, 50 μM (nucleotide concentration) single-stranded DNA template [M13mp8(t)], 9.4 units of primase activity, 1.8 units of polymerase α activity, 500 μM ATP, 200 μM UTP, 200 μM CTP, 5 μM dATP, 5 μM dGTP, 5 μM dCTP, and 5 μM [α-^{32}P]dTTP, plus various concentrations of GTP (0–500 μM), in a final volume of 10 μl. The samples were incubated at 37° for 60 min. The reactions were stopped by the addition of EDTA to a final concentration of 10 mM and were then immediately diluted with 3 volumes of electrophoresis loading buffer, which consisted of 98% (v/v) formamide, 10 mM EDTA, 0.01% (w/v) xylene cyanol, and 0.01% (w/v) bromphenol blue. The samples were then heated at 90° for 5 min and electrophoresed in a 10% polyacrylamide-8 M urea sequencing gel. Autoradiography was carried out at room temperature without intensifying screens. Size markers were digests of pBR322 plasmid with *Eco*RI (4300 base pairs) and *Eco*RI plus *Pst*I (3600 and 750 base pairs) and a ladder of repeating [5′-^{32}P]d(GACT)$_{8-32}$. The pBR322 fragments were labeled by filling in the 3′-termini with the Klenow fragment of DNA polymerase I (27). The amount of RNA-primed DNA was determined by scanning the dried gels with a PhosphorImager (Molecular Dynamics).

Results

Effects of mizoribine and mycophenolic acid on cell viability. Human leukemia CEM cells were incubated with either no drugs or increasing concentrations of either mizoribine or mycophenolic acid, and the number of viable cells was determined after 72 hr by trypan blue exclusion (Fig. 1). Mizoribine and mycophenolic acid induced a 50% reduction in the number of viable cells, compared with untreated controls, at concentrations of 4 μM and 0.5 μM, respectively. The IC_{50} values of mizoribine and mycophenolic acid determined with the MTT assay were 2 μM and 0.5 μM, respectively. Mizoribine (13) and mycophenolic acid (14) are inhibitors of IMP dehydrogenase, the enzyme that catalyzes the first reaction in the conversion of IMP to GMP. To determine whether the inhibition of IMP dehydrogenase and the consequent GTP depletion contributed to the antiproliferative effects of mizoribine and mycophenolic acid in CEM cells, drug-treated cells were coincubated with 50 μM guanosine and 100 μM 8-aminoguanosine to replenish intracellular GTP. By inhibiting purine nucleoside phosphorylase, 8-aminoguanosine slows the rate of conversion of guanosine to guanine, which results in a more sustained increase in the intracellular levels of GTP, compared with guanine alone (13). Loss of cell viability induced by either mizoribine or mycophenolic acid, at their respective IC_{50} levels, was completely prevented by coincubation with guanosine plus 8-aminoguanosine. It is evident from Fig. 1 that purine supplementation was less

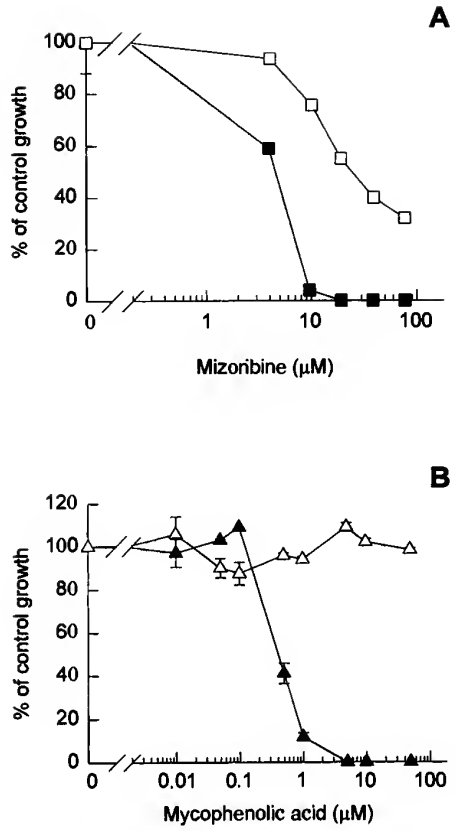

Fig. 1. Effects of mizoribine (A) and mycophenolic acid (B) on tumor cell viability. CEM cells were incubated with either no drug or increasing concentrations of either mizoribine alone (■), mycophenolic acid alone (▲), mizoribine plus 50 μM guanosine and 100 μM 8-aminoguanosine (□), or mycophenolic acid plus 50 μM guanosine and 100 μM 8-aminoguanosine (△). The numbers of viable cells were determined by trypan blue exclusion after 72 hr of continuous exposure to the compounds. Each *point* represents the mean ± standard deviation. Error bars were omitted when smaller than the size of the symbol.

effective at concentrations of mizoribine greater than 4 μM, which supports earlier observations that mizoribine at high concentrations is a less selective inhibitor of IMP dehydrogenase than is mycophenolic acid (12, 13). Therefore, the maximum concentration of mizoribine used in all subsequent experiments was 4 μM.

Induction of internucleosomal DNA degradation by mizoribine and mycophenolic acid as a function of time. Many cancer chemotherapeutic agents activate a program of cell death termed apoptosis (33). A characteristic feature of apoptosis is the appearance of DNA ladders of multiples of 180-base pair fragments that result from endonuclease digestion of DNA at internucleosomal sites. The goal of the studies described herein was to investigate some early biochemical effects of mizoribine and mycophenolic acid that might be causative, rather than reflective, of tumor cell death. Therefore, it was necessary to carry out experiments before the appearance of internucleosomal DNA degradation. Drug-induced apoptosis was evaluated by monitoring the formation of both nucleosomal and polynucleosomal DNA fragments. Groups of CEM cells were incubated for 0, 2, 4, and 16 hr with either 4 μM mizoribine or 1 μM mycophenolic

acid, and the low molecular weight DNA was isolated and purified. These DNA fragments were resolved in a 2% neutral agarose gel and stained with ethidium bromide. A nucleosomal ladder was not detected after a 2-hr incubation with either mizoribine or mycophenolic acid (Fig. 2A). However, a faint ladder was visible after 4 hr, which increased markedly in intensity after a 16-hr incubation of the cells with either compound. GTP depletion was responsible for the effects of both mizoribine and mycophenolic acid on DNA integrity. Repletion of the intracellular GTP pool with guanosine and 8-aminoguanosine completely prevented the formation of the nucleosomal ladder that was observed in cells treated with either compound alone for 4 or 16 hr (Fig. 2A).

It was possible that a 2-hr incubation of CEM cells with either mizoribine or mycophenolic acid induced only modest DNA fragmentation, which might not have been detected in the gel assay depicted in Fig. 2A. To examine high molecular weight DNA, additional groups of CEM cells were treated with either mizoribine or mycophenolic acid as described above. Drug-induced formation of higher molecular weight DNA fragments was then investigated by lysing the whole cells directly in the wells of a 2% agarose gel (30). This circumvented the need for DNA purification, which would likely have introduced additional DNA breakage. High molecular weight DNA fragments either remain in the wells of a 2% agarose gel or migrate a minimal distance into the gel. Untreated control cells and cells incubated for 2 hr with either mizoribine or mycophenolic acid contained only high molecular weight DNA and there was no evidence of DNA fragmentation (Fig. 2B). In contrast, lower molecular weight DNA fragments consisting of multimers of about 180 base pairs appeared in cells incubated with these compounds for 4 and 16 hr. Again, digestion of DNA to oligonucleosomal fragments was completely prevented by coincubation of the drug-treated cells with guanosine and 8-aminoguanosine. Based on these results, all subsequent experiments involving intact cells were carried out at 2 hr after drug treatment, when no DNA fragmentation was detected.

Effects of mizoribine and mycophenolic acid on the intracellular levels of GTP and ATP. Mizoribine and mycophenolic acid inhibit IMP dehydrogenase activity and, as a result, induce intracellular GTP depletion in human T lymphocytes (13, 14). As described above, the effects of either of these agents on cell viability and DNA integrity were prevented by coincubation of the cells with guanosine and 8-aminoguanosine. To demonstrate that drug-induced GTP depletion occurred in CEM leukemia cells before the onset of drug-induced DNA fragmentation, the levels of GTP and ATP were measured in duplicate after incubation of the cells for 2 hr with IC_{50} levels of these compounds. The mean GTP concentrations in cells incubated without drug or with 4 μM mizoribine or 0.5 μM mycophenolic acid were 7.7 (range, 6.7–8.7), 2.5 (range, 2.3–2.6), and 3.2 (range, 2.2–4.3) nmol/10^7 cells, respectively. Compared with the untreated control, this represents 68% and 58% depletion of GTP in cells treated with mizoribine and mycophenolic acid, respectively. In contrast, the intracellular concentration of ATP was not affected by either mizoribine or mycophenolic acid. ATP concentrations averaged 38 (range, 32–43), 37 (range, 32–41), and 35 (range, 32–37) nmol/10^7 cells in untreated control, mizoribine-treated, and mycophenolic acid-treated cells, respectively. A lack of effect of mizoribine or mycophenolic acid on the levels of ATP was also observed in human T lymphocytes (13, 14). The selective depletion of GTP was consistent with inhibition of IMP dehydrogenase by these agents and was similar in degree to that observed in a variety of other cell types (34).

Effects of mizoribine and mycophenolic acid on RNA, primer RNA, and DNA synthesis in CEM cells. Mizoribine or mycophenolic acid could inhibit DNA synthesis in several ways. The rNTP and dNTP levels in cells are

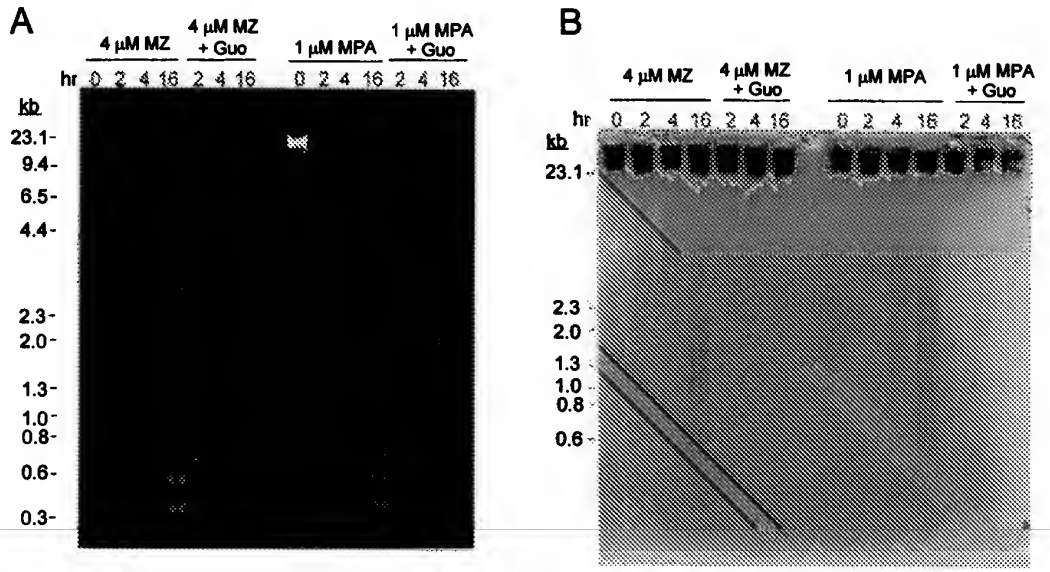

Fig. 2. Induction of internucleosomal DNA degradation by mizoribine or mycophenolic acid as a function of time. CEM cells were incubated for various times with either no drugs, 4 μM mizoribine (*MZ*) alone, 4 μM mizoribine plus 50 μM guanosine and 100 μM 8-aminoguanosine (*Guo*), 1 μM mycophenolic acid (*MPA*) alone, or 1 μM mycophenolic acid plus 50 μM guanosine and 100 μM 8-aminoguanosine. Low molecular weight DNA (A) and high molecular weight DNA (B) were analyzed as described in Experimental Procedures.

regulated in a coordinated fashion through multiple feedback controls (35). Thus, it was possible that the GTP depletion induced by mizoribine or mycophenolic acid resulted in a secondary depletion of a dNTP required for DNA synthesis. However, GTP depletion may directly lead to DNA synthesis inhibition. DNA synthesis on the lagging strand of the replication fork is discontinuous and involves the formation of RNA-primed DNA (Okazaki fragments). DNA primase, an RNA polymerase, utilizes rNTPs in the synthesis of the primer RNA component of the Okazaki fragments. Accordingly, a primary goal of the studies reported herein was to test the hypothesis that mizoribine and mycophenolic acid block DNA synthesis by depleting cells of the GTP required for primer RNA formation.

CEM cells were preincubated with [^{14}C]thymidine to uniformly label the DNA and were then treated for 2 hr with either 2 μM mizoribine or 0.5 μM mycophenolic acid. At the end of the 2-hr incubation, control and drug-treated cells were pulse-labeled with [^{3}H]adenosine. Radiolabeled RNA-primed DNA was separated from total RNA by centrifugation to equilibrium through two consecutive CsCl gradients (Fig. 3). To correct for any differences in the recoveries of the RNA-primed DNA among the various samples, the amount of [^{3}H]RNA-primed DNA present in the DNA density region of each second gradient was normalized to the amount of [^{14}C]DNA recovered from that gradient. Mizoribine (Fig. 3A) and mycophenolic acid (Fig. 3B) reduced the amount of [^{3}H]adenosine incorporated into the primer RNA to 25% and 30% of the untreated controls, respectively. The inhibition of primer RNA synthesis induced by either mizoribine or mycophenolic acid was prevented by coincubation of the cells with guanosine plus 8-aminoguanosine. Thus, mizoribine and mycophenolic acid can inhibit RNA-primed DNA synthesis in CEM cells by inducing GTP depletion.

Results from control experiments indicated that the ^{3}H present in the DNA density region of the gradient was actually incorporated into primer RNA. The labeled products were covalently linked to newly replicated DNA and migrated in polyacrylamide gels as oligomers of normal primer length (8–11 nucleotides) after digestion of the attached DNA with DNase I (data not shown). The products identified as primer RNA were also sensitive to alkaline hydrolysis.

Another possible explanation for these effects is that mizoribine and mycophenolic acid interfered with the uptake of [^{3}H]adenosine into the cellular acid-soluble pool, i.e., inhibited [^{3}H]adenosine transport or the anabolism of [^{3}H]adenosine to ribonucleotides. To address this possibility, the uptake of [^{3}H]adenosine into the acid-soluble pool in CEM cells was measured over a time course of 30 min, after incubation of the cells for 2 hr with either 2 μM mizoribine or 0.5 μM mycophenolic acid. [^{3}H]Adenosine uptake reached a steady state within 10 min in cells incubated with either no drug, mizoribine, or mycophenolic acid, and at this time the total intracellular concentrations of acid-soluble ^{3}H averaged 586 ± 32 μM, 505 ± 65 μM, and 542 ± 45 μM (mean ± standard deviation), respectively. These results indicate that the observed inhibition of [^{3}H]adenosine incorporation into primer RNA in drug-treated cells was not the result of decreased anabolism of [^{3}H]adenosine into acid-soluble nucleotides.

Both mizoribine (Fig. 4A) and mycophenolic acid (Fig. 4B) were more potent inhibitors of [^{3}H]adenosine incorporation into primer RNA than into total RNA. The degree of primer RNA synthesis inhibition produced by each agent was closely related to the extent of DNA synthesis inhibition (Fig. 4). These results further suggest that inhibition of RNA-primed DNA synthesis is an important mode of action of these compounds.

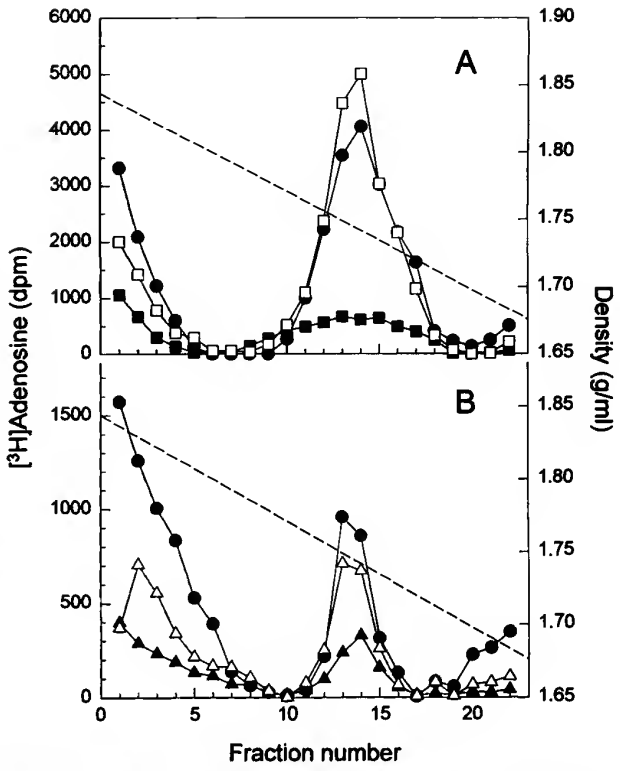

Fig. 3. Effects of mizoribine and mycophenolic acid on RNA-primed DNA synthesis. A, CEM cells were incubated with either no drug (●), 2 μM mizoribine alone (■), or 2 μM mizoribine and 50 μM guanosine plus 100 μM 8-aminoguanosine (□). B, CEM cells were incubated with either no drug (●), 0.5 μM mycophenolic acid alone (▲), or 0.5 μM mycophenolic acid and 50 μM guanosine plus 100 μM 8-aminoguanosine (△). At the end of a 2-hr incubation, all control and drug-treated cells were pulse-labeled with [^{3}H]adenosine. Primer RNA was isolated by centrifugation of the nucleic acids through two consecutive CsCl gradients. The distribution of radioactivity in the fractions of the second CsCl gradient is shown. The amount of ^{3}H radioactivity in the DNA density region of the gradient (1.72–1.75 g/ml) represents the amount of primer RNA synthesized in the cells.

Effects of GTP depletion on primer RNA synthesis in whole-cell lysates. To evaluate the effects of GTP depletion alone on primer RNA synthesis, we used a previously developed whole-cell lysate system in which RNA-primed DNA synthesis was dependent upon the addition of exogenous rNTPs and dNTPs (31). Lysates in one group contained all four rNTPs and dNTPs, whereas the lysates in a second group lacked exogenous GTP but contained all other rNTPs and dNTPs. RNA-primed DNA was labeled with [α-^{32}P]ATP and separated on 20% polyacrylamide gels (Fig. 5). After DNase I digestion to remove the attached DNA, the free primers synthesized in both the presence (Fig. 5, *lanes 1* and *2*) and the absence (Fig. 5, *lanes 3* and *4*) of GTP migrated as fragments of primarily 8–10 nucleotides in length. However, less primer RNA was synthesized in the lysates lacking GTP.

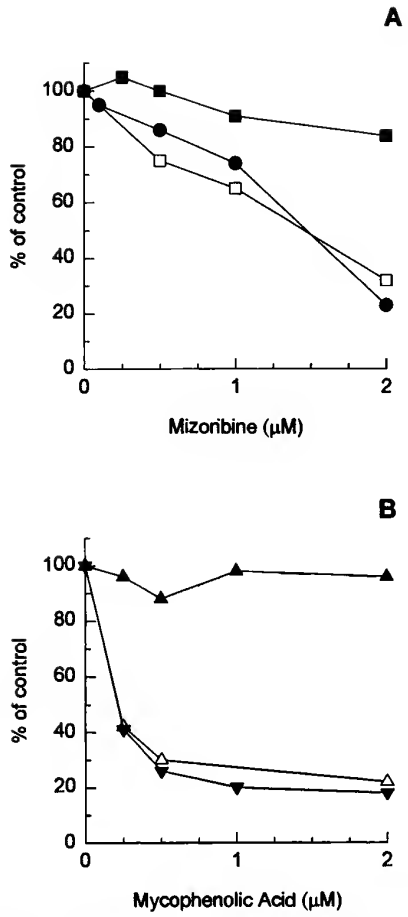

Fig. 4. Inhibition of RNA and RNA-primed DNA synthesis as a function of the concentration of either mizoribine or mycophenolic acid. CEM cells were incubated for 2 hr at 37° with the indicated concentrations of either mizoribine (A) or mycophenolic acid (B). Control and drug-treated cells were then resuspended in fresh medium and pulse-labeled with either [^{3}H]adenosine or [^{3}H]thymidine for 30 min at 37°. The amounts of [^{3}H]adenosine or [^{3}H]thymidine incorporated into total RNA and DNA were determined after acid precipitation of cellular macromolecules and either alkaline or acid hydrolysis of the RNA and DNA, respectively. The amount of [^{3}H]adenosine incorporated into primer RNA was measured after isolation of primer RNA by centrifugation through two consecutive CsCl gradients, as described in the legend to Fig. 3. [^{3}H]Adenosine incorporation into total RNA of cells treated with either mizoribine (■) or mycophenolic acid (▲), [^{3}H]adenosine incorporation into primer RNA of cells treated with either mizoribine (□) or mycophenolic acid (△), and [^{3}H]thymidine incorporation into DNA of cells treated with either mizoribine (●) or mycophenolic acid (▼) are shown. Control samples for primer RNA, total RNA, and DNA synthesis contained an average of 51,958, 57,883, and 43,228 dpm of ^{3}H, respectively. The standard deviation associated with each mean value was less than the size of the symbol.

Scanning of the corresponding areas of the autoradiographic film with a laser densitometer revealed that primer RNA synthesis was reduced to 16% of control (range, 14–18%) in the lysates lacking exogenous GTP. These results indicate that in a whole-cell lysate system GTP depletion alone can directly result in primer RNA synthesis inhibition.

Effect of GTP depletion on RNA-primed DNA synthesis catalyzed by the purified DNA primase-polymerase α complex. The results obtained with the cell lysate system supported the hypothesis that GTP depletion leads to inhibition of RNA-primed DNA synthesis. To further investigate the effects of GTP depletion on DNA primase activity, we measured RNA-primed DNA synthesis by the purified DNA polymerase α-primase complex as a function of GTP concentration. Because full length primer RNAs are only 8–10 nucleotides in length, the amount of radiolabeled rNTPs that can be incorporated into primer RNAs on an unprimed M13 DNA template is relatively small. To facilitate detection of the products on a sequencing gel, a coupled DNA primase-polymerase α assay was used to incorporate [^{32}P]dTTP into the nascent DNA attached to the primers. Under these conditions the amount of [^{32}P]dTTP incorporated into the RNA-primed DNA was dependent on the amount of primer RNA synthesized by the primase. The closed circular M13 DNA template cannot hybridize to itself to form primers with free 3′-OH ends (18, 20). Furthermore, in the absence of the four NTPs we observed no incorporation of [^{32}P]dTTP into the nascent DNA (data not shown).

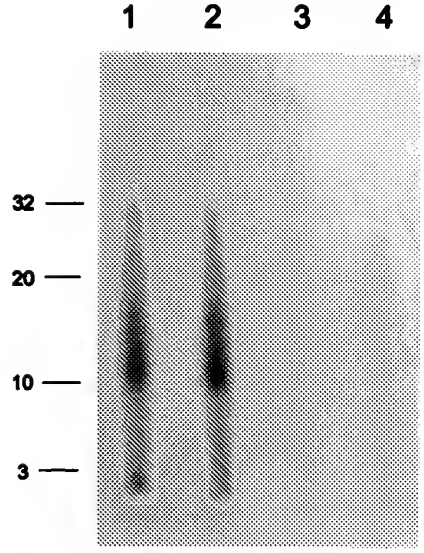

Fig. 5. Effects of GTP depletion on primer RNA synthesis in cell lysates. Whole-cell lysates prepared from exponentially growing CEM cells were incubated for 10 min with [^{32}P]ATP. The lysates were incubated in the presence (*lanes 1* and *2*) or absence (*lanes 3* and *4*) of 1 mM GTP plus all remaining rNTPs and dNTPs. Primer RNA was isolated as described and was analyzed by electrophoresis on a 20% polyacrylamide gel, as described in Experimental Procedures. *Numbers to the left of lane 1*, oligonucleotide size markers.

Fig. 6A, *lane 1*, indicates that in the absence of enzyme no RNA-primed DNA was synthesized. Fig. 6A, *lanes 2-6*, shows the products synthesized by the polymerase α-primase complex on the single-stranded M13 DNA template in the presence of decreasing concentrations of GTP (from 500 to 0 μM). Preliminary studies indicated that maximal RNA-primed DNA synthesis was observed with 500 μM GTP. The major products were about 750-3600 nucleotides in length, which is in good agreement with the results reported by others (20, 36). Reductions in the concentrations of GTP from 500 to 0 μM were accompanied by a progressive decrease in the amounts of RNA-primed DNA synthesized (Fig. 6A, *lanes 2-6*). Quantitative data were obtained by scanning with a PhosphorImager the gels obtained from two separate experiments and analyzing the digitized images with Image-Quant software (Fig. 6B). Total RNA-primed DNA synthesis was

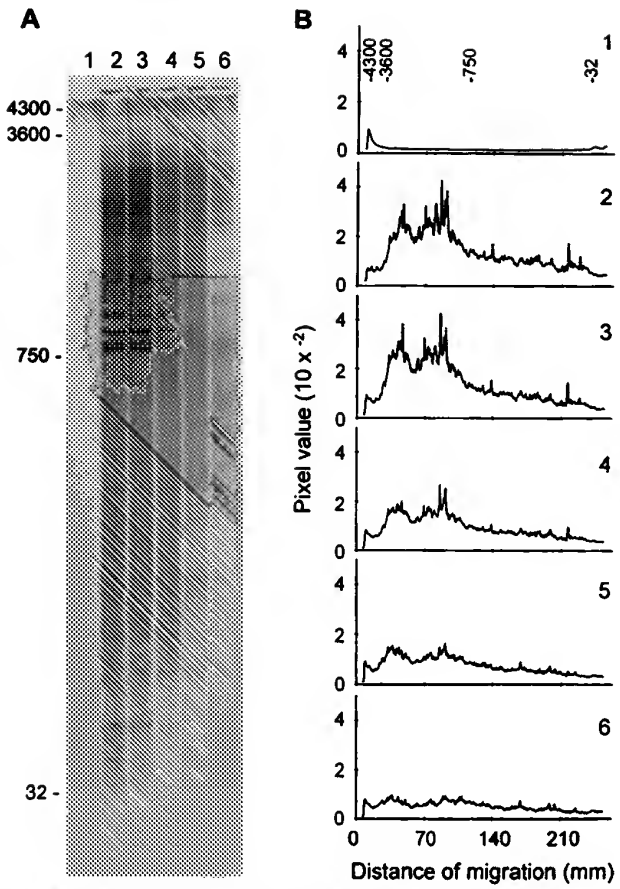

Fig. 6. Effects of GTP concentration on RNA-primed DNA synthesis by the purified DNA polymerase α-primase complex. A, Primase-dependent polymerase α activity was assayed in the presence of 30 μg/ml single-stranded M13mp8(+) DNA, 500 μM ATP, 200 μM CTP, 200 μM UTP, 5 μM dATP, 5 μM dCTP, 5 μM dGTP, 5 μM [^{32}P]dTTP, and the indicated concentrations of GTP. The samples were incubated for 60 min at 37° and then electrophoresed on a sequencing gel. *Numbers to the left of lane 1*, nucleotide size markers. *Lane 1*, complete reaction mixture containing 500 μM GTP but no enzyme; *lanes 2-6*, samples containing 500, 250, 125, 25, and 0 μM GTP, respectively. B, The sequencing gel shown in A was scanned with a PhosphorImager and the digitized images were analyzed and plotted with Image-Quant software. *Numbers in the upper right corners*, corresponding lanes in the sequencing gel.

decreased by an average of 4% (range, 1–8%), 33% (range, 32–34%), 51% (range, 49–52%), and 70% (range, 69–71%) in reactions containing 250, 125, 25, and 0 μM GTP (Fig. 6B, *3-6*), respectively, compared with that in the reaction containing 500 μM GTP (Fig. 6B, *2*). The effect of decreasing GTP concentrations was particularly evident with the major products in the size range of 750-3600 nucleotides.

In addition to decreasing the synthesis of RNA-primed DNA, GTP depletion altered the pattern of RNA-primed DNA fragments that was observed on the sequencing gels. This was observed primarily with the lower molecular weight fragments (32–750 nucleotides). For example, with 25 and 0 μM GTP (Fig. 6A, *lanes 5* and *6*, respectively) several bands were visible that were not apparent in the reaction containing 500 μM GTP (Fig. 6A, *lane 2*). The most likely explanation for this phenomenon is that GTP depletion affects the selection of the primase initiation sites such that primers are less likely to be initiated opposite dCMP sites in the template.

Discussion

The studies described herein demonstrate that incubation of CCRF-CEM cells for 2 hr with IC_{50} levels of either mizoribine or mycophenolic acid results in about 60–70% depletion of total intracellular GTP, without affecting the ATP pool. Similar results were obtained when various other cell lines were treated with these drugs (2, 3). One possible mechanism whereby guanine nucleotide depletion could directly lead to inhibition of DNA replication is by preventing normal rates of primer RNA synthesis catalyzed by DNA primase. The degree of primer RNA synthesis inhibition induced by a brief incubation (2 hr) of CEM cells with either mizoribine or mycophenolic acid was similar to the degree of DNA synthesis inhibition over the entire range of drug concentrations tested. It is especially noteworthy that primer RNA synthesis inhibition was reversible with repletion of the guanine nucleotide pools, establishing a causal relationship between these events.

To determine whether this brief period of guanine nucleotide reduction had resulted in DNA fragmentation that could have secondarily affected either primase or polymerase activity, we examined both low and high molecular weight DNA for evidence of internucleosomal cleavage compatible with the induction of apoptosis. We found that IMP dehydrogenase inhibition did induce a limited amount of apoptosis after 4 hr, with a much more pronounced effect at 16 hr. Again, this phenomenon was prevented by repletion of the guanine nucleotide pools by coincubation of the cells with guanosine and 8-aminoguanosine. Of particular importance, however, was the lack of DNA degradation at the 2-hr time point, at which the RNA primer synthesis studies were performed. Thus, these data support the conclusion that the depletion of GTP and subsequent inhibition of primer RNA synthesis are early drug effects that interfere with DNA synthesis.

Other lines of evidence are consistent with our observations that primer RNA synthesis is highly sensitive to intracellular GTP depletion. The half-lives of primer RNAs are very short (seconds to minutes), compared with those of mRNA and other RNAs (hours to days) (32). RNA primers isolated from whole cells have either GTP or ATP almost exclusively at their 5′ termini (22–24). On single-stranded natural DNA templates the purified primase likewise prefers to initiate primer synthesis with GTP or ATP, rather than with a pyrimidine rNTP (17–21), and, furthermore, prefers to utilize GTP as the second nucleotide (37). The importance of GTP in primer RNA synthesis stems from the fact that the additional hydrogen bond of the G-C base pair enhances the stability of the dinucleotide-template complex (37). This in turn increases the probability that the dinucleotide will be elongated to full length primer RNA, rather than prematurely dissociating from the DNA template.

It has been reported that mycophenolic acid indirectly induces intracellular dGTP depletion (3), which may lead to inhibition of DNA synthesis without involving primer RNA formation. At this time it is not possible to state unequivocally that depletion of GTP, as opposed to dGTP, is primarily responsible for inhibition of DNA synthesis. A thorough eval-

uation of the relative importance of GTP versus dGTP depletion would require knowledge of the concentrations of these nucleoside triphosphates that are available at the replication fork for either primer RNA or DNA synthesis. Unfortunately, it is very difficult to accurately estimate these concentrations. Considerable evidence indicates that quantitation of total intracellular nucleotide pools with standard perchloric acid or methanol extraction techniques does not take into account the probable compartmentalization of either rNTPs or dNTPs (10). However, our studies using either a whole-cell lysate system or the purified DNA polymerase α-primase complex indicate that GTP depletion alone is capable of blocking RNA-primed DNA synthesis in the presence of all other rNTPs and dNTPs, including dGTP. It has previously been demonstrated that S49 cells deficient in hypoxanthine-guanine phosphoribosyltransferase activity are unable to salvage guanine to GMP (8, 9). In the S49 mutants incubated with mycophenolic acid, 2′-deoxyguanosine replenished the dGTP pool but not the GTP pool and was unable to completely restore DNA synthesis. Thus, these data, when taken together with the results presented in this communication, support a direct role for GTP depletion in the cytotoxicity that results from IMP dehydrogenase inhibition.

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